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Environmental Isolates of Azole-Resistant *Aspergillus fumigatus* in Germany

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Azole antifungal drug resistance in *Aspergillus fumigatus* is an emerging problem in several parts of the world. Here we investigated the distribution of such strains in soils from Germany. At a general positivity rate of 12%, most prevalently, we found strains with the TR_{34}/L98H and TR_{46}/Y121F/T289A alleles, dispersed along a corridor across northern Germany. Comparison of the distributions of resistance alleles and genotypes between environment and clinical samples suggests the presence of local clinical clusters.

Since the mid-1990s, a steady increase in the occurrence of itraconazole-resistant *Aspergillus fumigatus* has been observed in clinical contexts (1) and has been linked to therapeutic failure in the treatment of aspergillosis (2). *A. fumigatus* conidia are ubiquitously found in the environment; there, habitats of *A. fumigatus* include those with elevated temperatures, e.g., compost heaps. This allows this species to successfully infect immunity-deficient warm-blooded animals, including humans. Since there is no reservoir in healthy hosts, infections are generally thought to be acquired exogenously from the environment. Clinical manifestations range from pulmonary colonization and deep invasive mycoses of the lung and other tissues to fatal sepsis in immunocompromised patients. Only a limited number of antifungal drugs are available for therapy, among which azoles are inhibitors of the Cyp51A protein, a central enzyme in the ergosterol biosynthesis pathway. Several *cyp51A* mutations have become known that lead to decreased drug susceptibility *in vitro* and possibly to therapy failure in patients. These mutations are thought to arise under conditions of prolonged antifungal therapy or prophylaxis in individual patients (3).

The recent increase in azole resistance in *A. fumigatus*, however, has been linked to two *cyp51A* alleles, termed "TR_{34}/L98H" and "TR_{46}/Y121F/T286A." These combinations of promoter tandem repeats and amino acid exchanges are thought to have arisen through the use of agricultural fungicides which are structurally similar to clinically used azoles (4, 5). Apparently, these alleles are now spreading, since they have been reported over recent years to occur in clinical and environmental isolates collected across Eurasia, including Germany (6–9), and Africa (10) but not (yet) North America (11) within different genetic backgrounds.

We investigated whether isolates with the predominant resistance alleles found in German patients are also present in the environment with a similar frequency. During the summers of 2012 and 2013, 455 soil samples were obtained and screened for the presence of itraconazole-resistant or voriconazole-resistant *A. fumigatus* strains. Approximately 1 ml of each sample was subjected to thorough vortex mixing in 5 ml 0.5% (wt/vol) saponin, the debris was briefly allowed to settle, and the supernatant was transferred to a fresh tube. The resulting suspension was centrifuged and the pellet resuspended in a final volume of 500 μl sterile 0.9% (wt/vol) NaCl. A 100-μl volume (each) was plated on Sabouraud agar containing no drug or 1 $\mu g \cdot ml^{-1}$ itraconazole or 1 $\mu g \cdot ml^{-1}$ voriconazole (both from Discovery Fine Chemicals, Bournemouth, United Kingdom). Each sample was processed in three biologically independent experiments. Colonies growing after 2 to 4 days were subcultured and their species determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (MALDI Biotyper, Bruker Daltonics, Bremen, Germany). Susceptibility to itraconazole, voriconazole, and posaconazole was tested by EUCAST (12) broth microdilution (Table 1). For both environmental (Table 2) and clinical (see Table S1 in the supplemental material) resistant isolates (6–9, 13), the *csp1* types were determined to estimate genetic diversity (14–17).

Using this procedure, a total of 55 resistant isolates were recovered (Table 1) and subjected to sequencing of the *cyp51A* gene.

As expected, the majority of resistant strains harbored the TR_{34}/L98H allele ($n = 45$), which is also the allele most frequently observed in clinical isolates from Germany (8, 9, 13) (Table S1 in the supplemental material). One isolate displayed an unusually high voriconazole MIC_0 of >32 $\mu g \cdot ml^{-1}$, indicating the presence of an additional, non-Cyp51A-based resistance mechanism.

Most TR_{34}/L98H strains from both clinical and environmental sources formed a distinct group (type t04B) which is not frequently found in susceptible isolates (Table 2). Clinical t04B isolates were exclusive to the Rhineland area (Cologne, Essen, Düsseldorf). A second smaller local cluster was observed with three clinical t02 isolates from Munich, a type which was not frequently found among environmental isolates.

Second most frequently, we observed the TR_{46}/Y121F/T289A

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TABLE 1 Drug resistance patterns

Cyp51A isoform	*n*	MIC_0 range ($\mu g \cdot ml^{-1}$)		
		Itraconazole	Voriconazole	Posaconazole
TR_{34}/L98H	45	>32	1 to 4 and >32[a]	0.125 to 0.5
TR_{46}/Y121F/T289A	5	1 to 2	4 to >32	1
TR_{46}/Y121F/M172I/T289A	1	1	>32	0.5
G54A	2	>32	0.125	1
M220I	1	>32	1	0.5
Wild type	1	>32	8	1

[a] Forty-four isolates with MIC_0 values within the range of 1 to 4, and one isolate at >32.

variant ($n = 6$). One isolate additionally had a M172I substitution, and such a strain has subsequently been observed in a leukemia patient from Dresden (S. Rößler and O. Bader, unpublished results). In Germany, the TR_{46}/Y121F/T289A allele has been described only recently in isolates from cystic fibrosis and stem cell transplant patients (9, 13) but has previously been documented in isolates from the environment in neighboring countries (5, 18, 19). Isolates with TR_{46}/Y121F/T289A have uniformly been linked to therapeutic failure for treatment of invasive aspergillosis (5, 18).

Conidiation of *A. fumigatus* is observed only rarely within tissues, and no data exist on how resistant isolates might spread between patients or even from patients to the environment. It was therefore surprising to observe environmental isolates with the clinically well-known M220I and the novel G54A substitutions. These have been proposed to emerge under conditions of prolonged therapy (5), but their presence in the environment may also argue for a possible agricultural origin. Although the environmental and clinical M220I isolates were not genetically linked (type t03 versus t01), this hypothesis is supported by the recovery of an M220L isolate from an azole-naive cystic fibrosis patient (9), where it may constitute a transient colonizer. Together, these data suggest that environmental spread is also a possibility.

Finally, we observed one resistant isolate without any alteration of the *cyp51A* gene (type t03). Resistant isolates without changes in *cyp51A* are frequent in patients (8, 13, 20) but also occur in the environment (21). Typing of the respective clinical isolates showed that they were of types t01, t02, and mostly t03, which again may indicate an exchange between the environment and patients.

Looking at the prevalence of azole-resistant *A. fumigatus* isolates across Europe from the north to the south, resistant strains have not been found in the environment in Denmark (19), despite the fact that both TR_{34}/L98H- and TR_{46}/Y121F/T286A-carrying strains have been isolated from patients there (19, 22). Similarly, the environmental prevalence of resistant strains in the United Kingdom is low (21). This is in agreement with the lower numbers of resistant isolates in the northern part of Germany (region I; see Fig. S1 and Table S2 in the supplemental material) seen here. The prevalence of isolates with TR_{34}/L98H or TR_{46}/Y121F/T289A alleles was highest toward the center of Germany (region III).

An absence of resistant strains was evident in southern Germany, despite the fact that we had previously seen resistant isolates in clinical specimens (8). This was in agreement with a previous environmental study in Austria, where no resistant isolates were

TABLE 2 *csp1* subtypes of drug-resistant *A. fumigatus*

Origin and Cyp51A isoform	Isolate category[a]	Total no. of isolates	% isolates of indicated *csp1* subtype[b]									Reference(s) or source
			t01	t02	t03	t04A	t04B	t06B	t08	t11	Other	
Germany												
TR_{34}/L98H	C	12		25		13	50			17		6–9
	E	45		16			71			13		This study
TR_{46}/Y121F/T289A	C	1	100									9
	E	5	20			80						This study
TR_{46}/Y121F/M172I/T289A	C	1	100									Unpublished
	E	1	100									This study
G54A	C	0										
	E	2			100							This study
G54W	C	1	100									8
	E	0										This study
F219C	C	1	100									8
	E	0										This study
M220I	C	1	100									9
	E	1			100							This study
M220L	C	1			100							8
	E	0										This study
Wild type	C	9	22	11	44			11	11			8, 9, 13
	E	1			100							This study
Other countries												
Susceptibility and Cyp51A isoform unknown	C	492	26	9	17	23		2		1	22	14–17
	E	136	23	7	15	37					18	14, 17

[a] C, clinical strains (analyses of isolates were taken from references 6–9; details are given in Table S1 in the supplemental material); E, environmental strains.
[b] The nomenclature used in reference 16 was adapted according to Klaassen et al. (14). No discrimination of A and B subtypes for t04 given in reference 16; however, type t04A was indirectly suggested by Klaassen et al. (14).

found either (22). Further south, in northern Italy, TR_{34}/L98H-carrying strains are at least present again (23).

Taking the data together, the geographical distribution suggests the presence of a west-east distribution of TR_{34}/L98H and TR_{46}/Y121F/T289A isolates in both clinical (6–9, 13) and environmental samples, peaking in the middle of Germany. This might be explained by dispersion originating from the Netherlands, as suggested before (24). The increased prevalence of specific *csp1* types among TR_{34}/L98H isolates in Munich and Rhineland also suggests the presence of local factors that contribute to the epidemiology.

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O.B., M.W., and U.G. conceived the study. O.B., J.T., and M.T. performed the experiments. O.B., A.D., M.W., and U.G. wrote the manuscript.

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